



# *Luteimonas granuli* sp. nov., Isolated from Granules of the Wastewater Treatment Plant

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Received: 1 October 2018 / Accepted: 29 May 2020 / Published online: 5 June 2020  
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## Abstract

A Gram-reaction negative, aerobic, non-motile, light yellow colored, and rod-shaped bacterium (designated Gr-4<sup>T</sup>) isolated from granules of a wastewater treatment plant, was characterized by a polyphasic approach to clarify its taxonomic position. Strain Gr-4<sup>T</sup> was observed to grow optimally at 30 °C and at pH 7.0 on R2A medium. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain Gr-4<sup>T</sup> belongs to the genus *Luteimonas* of the family *Xanthomonadaceae* and was most closely related to *Luteimonas padinae* CDR SL 15<sup>T</sup> (99.1%), *Luteimonas terricola* DSM 22344<sup>T</sup> (98.5%) and *Luteimonas arsenica* 26-35<sup>T</sup> (97.6). The genome comprises 2,917,404 bp with a G+C content of 70.5 mol%. The ANI value between strain Gr-4<sup>T</sup> and *Luteimonas padinae* CDR SL 15<sup>T</sup> was 87.3%. The DNA–DNA relatedness value between strain Gr-4<sup>T</sup> and *Luteimonas padinae* CDR SL 15<sup>T</sup>, *Luteimonas terricola* DSM 22344<sup>T</sup> was 36.4 ± 1.3% and 14.2 ± 1.7%, respectively. The predominant quinone was Q-8. The major fatty acids were iso-C<sub>15:0</sub>, iso-C<sub>16:0</sub> and summed feature 9 (comprising iso-C<sub>17:1</sub> ω<sub>9c</sub> and/or C<sub>16:0</sub> 10-methyl) supported the affiliation of strain Gr-4<sup>T</sup> to the genus *Luteimonas*. Moreover, the physiological, biochemical results, and low level of ANI and DNA–DNA relatedness value allowed the phenotypic and genotypic differentiation of strains Gr-4<sup>T</sup> from other *Luteimonas* species with validly published names. The novel isolate therefore represents a novel species, for which the name *Luteimonas granuli* sp. nov. is proposed, with the type strain Gr-4<sup>T</sup> (=KACC 16614<sup>T</sup>=JCM 18203<sup>T</sup>).

## Introduction

The genus *Luteimonas* was described by Finkmann et al. [7] with a single species, *Luteimonas mephitis*, isolated from a biofilter. Phylogenetically, the genus is a member of

phylum *Proteobacteria* and closely to the genera *Lysobacter*, *Xanthomonas*, *Pseudoxanthomonas* and *Stenotrophomonas*, which share some characteristics such as the presence of branched-chain fatty acids and ubiquinone-8 (Q-8). Members of the genus are Gram-negative, non-flagellated, non-spore-forming, aerobic, rod-shaped, and light yellow colored on R2A, TSA, or NA agar plates [7, 24]. Species of the genus contained iso-C<sub>15:0</sub>, iso-C<sub>15:1</sub>, iso-C<sub>17:1</sub> ω<sub>7c</sub> and iso-C<sub>17:0</sub> as major fatty acids [7, 15]. Affiliates of this genus have been isolated from various sources including biofilter, tidal flat [16], fresh water, food waste, cucumber leaf, seawater, stratum water, sediment, and soil. At the time of writing, the genus *Luteimonas* comprises 19 recognized species (<https://www.bacterio.net>) with recently described species *Luteimonas notoginsengisoli* [3] *Luteimonas terrae* [14], *Luteimonas dalianensis* [24], and *Luteimonas lutimaris* [15].

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## Materials and Methods

### Isolation of Bacterial Strain

In this study, a novel bacterial strain was isolated from the granules of wastewater treatment plant in Daejeon city, Republic of Korea. The samples of granules from wastewater treatment plant were collected and the strain Gr-4<sup>T</sup> was isolated on R2A agar medium at 30 °C after 2 weeks of incubation. Strain Gr-4<sup>T</sup> was cultured routinely on R2A agar plates at 30 °C and preserved as a suspension in R2A broth supplemented with 20% (v/v) glycerol at –80 °C.

The reference strains *Luteimonas padinae* KCTC 52403<sup>T</sup>; 3, *Luteimonas terricola* DSM 22344<sup>T</sup>; 4, *Luteimonas arsenica* KCTC 42824<sup>T</sup> were collected from two different cultures [Korean culture collection (KCTC) and German culture collection (DSM), which were used as a references for comparison with the novel isolated strain.

### Phylogenetic Tree Construction

Genomic DNA of strain Gr-4<sup>T</sup> was extracted with a commercial genomic DNA extraction kit (Solgent, Korea) and PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Kim et al. [11]. Nearly full-length sequence of the 16S rRNA gene was compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of 33 related taxa were obtained from GenBank database [<https://www.ncbi.nlm.nih.gov/genbank>] or [<https://www.ezbiocloud.net/eztaxon>]. Multiple alignments were performed by Clustal\_X program [21]. Gaps were edited in the BioEdit program [9] and 1,375 nucleotides were used for phylogenetic tree construction. Evolutionary distances were calculated using the Kimura two-parameter model [12]. Phylogenetic trees were constructed using a neighbor-joining method [17], maximum-parsimony [8], and maximum-likelihood method [6] using the MEGA6 program [20] with bootstrap values based on 1000 replications [5].

### Complete Genome Sequencing and DNA G+C Content Analysis

Complete genome sequence of strain Gr-4<sup>T</sup> was determined by Illumina HiSeq X Ten and was assembled by SOAPdenovo v. 3.10.1 de novo assembler. The genome annotation was performed by the NCBI prokaryotic genome annotation pipeline (PGAP). To estimate the degree of pairwise relatedness between Gr-4<sup>T</sup> and *Luteimonas padinae* CDR SL 15<sup>T</sup>, BLAST-based average nucleotide identity (ANI) and

the genome-to-genome distance calculation (GGDC) were calculated as described previously [25].

The DNA G+C content (mol%) was analyzed via the complete genome sequence of the novel isolate using the online web server of NCBI.

### DNA–DNA Hybridization

Using photobiotin-labeled DNA probes and micro-dilution wells plate method, the DNA–DNA hybridization experiment was performed between strain Gr-4<sup>T</sup> and two reference strains *Luteimonas padinae* KCTC 52403<sup>T</sup> and *Luteimonas terricola* DSM 22344<sup>T</sup> [4].

### Physiological and Biochemical Characteristics

The Gram reaction was determined using the non-staining method, as described by Buck [1]. Cells motility was determined using hanging drop method, while cell morphology was examined with the scanning electron microscope (Hitachi SU-3500), using cells grown for 1 day at 30 °C on R2A agar medium. Catalase and oxidase tests were performed as outlined by Cappuccino and Sherman [2]. Biochemical tests were carried out using API 20NE, 32GN and API ZYM kits according to the instructions of the manufacturer (bioMérieux). Tests for degradation of DNA, casein, starch and carboxyl methyl cellulose were performed and evaluated after 7 days at 30 °C. Growth at different temperatures (4–45 °C, at intervals of 3 and 5 °C) and various pH values (pH 4–10 at intervals of 1 pH units) was assessed after 5 days incubation at 30 °C. Salt tolerance test was evaluated on R2A medium supplemented with 0–6% (w/v at intervals of 0.5% unit) NaCl and growth assessed after 7 days of incubation at 30 °C. Growth on different media were also tested using nutrient agar (NA, Difco), trypticase soy agar (TSA, Difco), LB agar (Difco) and MacConkey agar (Difco) at 30 °C. To determine the ginsenosides conversion ability of the novel isolate, the biotransformation of ginsenoside was carried out as described previously [19].

### Chemotaxonomy

#### Analysis of Respiratory Quinone and Cellular Fatty Acids

Isoprenoid quinone was extracted with chloroform/methanol (2:1, v/v), evaporated under vacuum condition, and re-extracted in *n*-hexane/water (1:1, v/v). The crude *n*-hexane-quinone solution was purified using Sep-Pak Vac cartridges silica (Waters) and subsequently analyzed by HPLC as previously described [10]. Cellular fatty acids profiles were determined for strains grown on R2A agar for 2 days at 30 °C. The cellular fatty acids were saponified, methylated, and extracted according to the protocol of the Sherlock Microbial

Identification System (MIDI). The fatty acids analyzed by a gas chromatograph (Hewlett Packard 6890) were identified by the Microbial Identification software package based on Sherlock Aerobic Bacterial Database (TSBA60) [20].

### Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequence and complete genome accession number for strain Gr-4<sup>T</sup> is JQ349045, and CP042218, respectively.

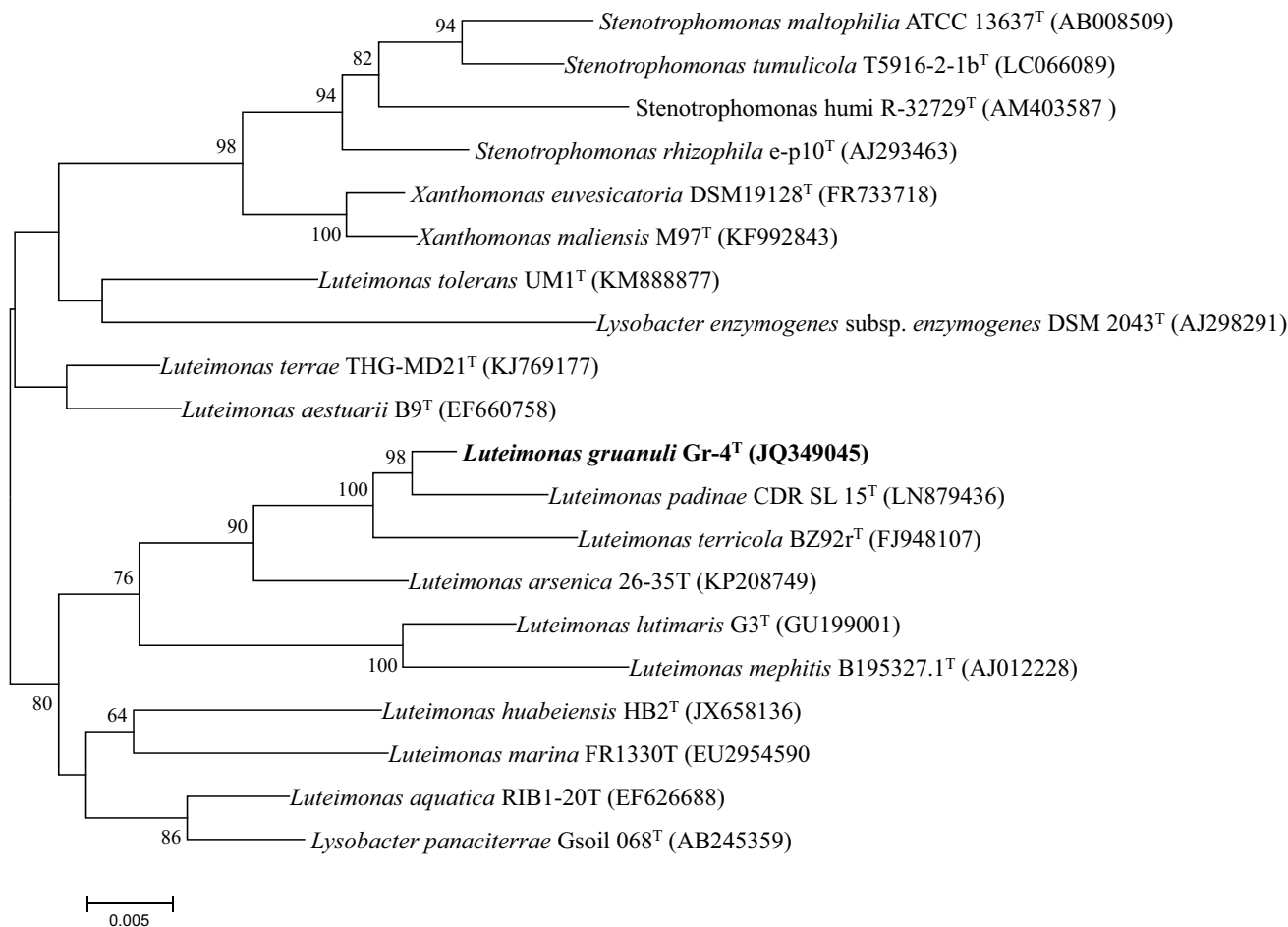
## Results and Discussion

### Phylogenetic Assessment and DNA G+C Content

The nearly complete 16S rRNA gene sequence of strain Gr-4<sup>T</sup> (1469 nt) was determined and subjected to

comparative analysis. Phylogenetic analysis using the maximum-likelihood method based on 16S rRNA gene sequences indicated that strain Gr-4<sup>T</sup> are clustered within the genus *Luteimonas* (Fig. 1). Moreover, this relationship was also evident in phylogenetic trees based on the neighbor-joining and maximum-parsimony methods.

Based on EzTaxon-e server (16S rRNA gene sequence similarity), strain Gr-4<sup>T</sup> show the highest sequence similarity with *Luteimonas padinae* KCTC 52403<sup>T</sup> (99.1%), *Luteimonas terricola* DSM 22344<sup>T</sup> (98.5%) and *Luteimonas arsenica* KCTC 42824<sup>T</sup> (97.6%), while share less than 96.7% 16S rRNA gene sequence resemblance with the type specie (*Luteimonas mephitis* DSM 12574<sup>T</sup>) and other members of the genus. Similarly, the topology of other trees based on maximum-likelihood, neighbor-joining and maximum parsimony analysis indicated that the novel isolate form a clad with *Luteimonas padinae*



**Fig. 1** Phylogenetic relationship of strain Gr-4<sup>T</sup>, recognized *Luteimonas* species and related species. The tree was constructed using the neighbor-joining method based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) greater than 60% are shown at branch points. Filled circles indicate

that the corresponding nodes were also recovered in the tree generated with maximum-parsimony and maximum-likelihood algorithms. Bar, 0.005 substitutions per nucleotide position. *Solimonas flava* DSM 18980<sup>T</sup> (EF154515) was used as an outgroup

KCTC 52403<sup>T</sup>, *Luteimonas terricola* DSM 22344<sup>T</sup> 52403<sup>T</sup> (99.1%) and *Luteimonas arsenica* KCTC 42824<sup>T</sup>.

The DNA G+C contents of strain Gr-4<sup>T</sup> was 70.2 mol%. The DNA G+C content was within the range to the other describe species of the genus *Luteimonas* (Table 1).

Complete genome sequence analysis show a total length of 2.92 Mb. Gene prediction has also been performed and 2481 protein coding genes, 53 RNA gene, 3 rRNA and 46 tRNA genes were detected by NCBI IMG/ER (<https://img.jgi.doe.gov/cgi>). The genomic DNA G+C content of strain Gr-4<sup>T</sup>, directly calculated from its genome sequence, was determined to be 70.5 mol% which was in the range of the previously described species of the genus *Luteimonas*. Furthermore the genome features of strain GR-4<sup>T</sup> are show in Table 2. The genomic ANI values between strain GR-4<sup>T</sup> and *Luteimonas padinae* KCTC 52403<sup>T</sup> was 87.3%. The low ANI value supports the characterization of strain Gr-4<sup>T</sup> as a novel species of the genus *Luteimonas*.

DNA–DNA relatedness value between strain Gr-4<sup>T</sup> and *Luteimonas padinae* KCTC 52403<sup>T</sup> and *Luteimonas terricola* DSM 22344<sup>T</sup> were  $36.4 \pm 1.3\%$  ( $34.2 \pm 1.8\%$ , reciprocal) and  $14.2 \pm 1.7\%$  ( $16.1 \pm 1.9\%$ , reciprocal), respectively. According to Wayne et al. [23], DNA–DNA relatedness values lower than 70% are considered to be the threshold value for the delineation of genospecies, so the result obtained is low enough to assign strain Gr-4<sup>T</sup> as a novel species of the genus *Luteimonas*.

## Phenotypic and Biochemical Tests

Cells of strain Gr-4<sup>T</sup> were Gram-reaction-negative, aerobic, non-motile, non-spore-forming, and rod-shaped. Colonies of strain Gr-4<sup>T</sup> grown on R2A agar were circular, convex, opaque, and light yellow color after 24 h incubation at 30 °C. The isolate did not grow on MacConkey agar (Difco), DNase agar (Difco) and LB (Difco), whereas weakly grow on nutrient agar (Difco) and TSA (Difco) at 30 °C. Physiological characteristics of strain Gr-4<sup>T</sup> is summarized in the species description and a comparison of selective characteristics of the isolated strain and related type strains are given in Table 1.

## Quinone, Polar Lipid, and Fatty Acids

The major respiratory quinone was ubiquinone Q-8. The fatty acid profile of strain Gr-4<sup>T</sup> was compared with those of the type strains of recognized *Luteimonas* species. The major fatty acids of strain Gr-4<sup>T</sup> were iso-C<sub>15:0</sub> (28.6%), iso-C<sub>16:0</sub> (15.2%), and summed feature 9 [(comprising iso-C<sub>17:1</sub> ω9c and/or C<sub>16:0</sub> 10-methyl) 13.5%], which is a typical profile of members of the genus *Luteimonas* [7]. However, some qualitative and quantitative variances in the fatty acids

**Table 1** Characteristics that differentiate *Luteimonas granuli* sp. nov. from its closely related species of the genus *Luteimonas*

Characteristics	1	2	3	4
Isolation source	Granules	Seaweed	Soil	Soil
Temperature range growth (°C)	18–42	15–40 <sup>a</sup>	1–25 <sup>b</sup>	10–42 <sup>c</sup>
NaCl range (%) for growth	0–3	0–4 <sup>a</sup>	0–3 <sup>b</sup>	1–5 <sup>c</sup>
Enzymes activity				
Acid phosphatase	+	–	–	+
α-Chymotrypsin	+	–	+	–
β-Glucuronidase	–	–	+	–
α-Glucosidase	–	–	+	–
β-Glucosidase	–	+	–	+
Nitrate reduction	+	+	–	–
Trypsin	+	–	–	+
Urease	+	+	–	–
Assimilation of				
Acetate	–	+	+	–
L-Arabinose	–	+	–	+
Adipate	+	+	–	–
N-Acetyl-D-glucosamine	–	–	–	+
Gluconate	–	+	+	–
D-Glucose	+	+	–	–
Glycogen	+	+	–	–
L-Histidine	–	+	+	+
3-Hydroxy-benzoate	–	–	+	–
Itaconate	–	–	+	–
2-Ketogluconate	–	+	–	+
5-Ketogluconate	–	–	–	+
Malate	–	–	+	+
Malonate	–	–	+	+
D-Melibiose	–	–	+	–
Phenyl-acetate	+	–	–	–
Propionate	–	–	+	+
L-Proline	–	–	+	+
D-Ribose	–	–	+	+
Salicin	+	–	+	–
D-Sorbitol	–	–	+	+
Suberate	–	+	+	+
Valerate	–	–	+	+
DNA G+C content (mol%)	70.5	73.4 <sup>a</sup>	72.0 <sup>b</sup>	71.4 <sup>c</sup>

1, Gr-4<sup>T</sup>; 2, *Luteimonas padinae* KCTC 52403<sup>T</sup>; 3, *Luteimonas terricola* DSM 22344<sup>T</sup>; 4, *Luteimonas arsenica* KCTC 42824<sup>T</sup>. All strains were positive for esculin hydrolysis, β-galactosidase, alkaline phosphatase, esterase, esterase Lipase, naphthol-AS-BI-phosphohydrolase and caprate. All strains were negative for indole production, arginine dihydrolase, gelatin hydrolysis, lipase, α-galactosidase, β-galactosidase, valine arylamidase, cystine arylamidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, glucose acidification, D-mannose, D-mannitol, D-maltose, caprate, citrate, D-glucose, L-fucose, 3-hydroxy-butyrate, 4-hydroxy-benzoate, L-rhamnose, inositol, D-sucrose, lactate, L-alanine and L-serine. the Gram reaction, spore formation and motility, but positive for oxidase/catalase reaction. +, positive; –, negative

<sup>a,b,c</sup>Data are taken from Verma et al. [22], Zhang et al. [26] and Mu et al. [13]

**Table 2** The genome features of strain *Luteimonas granuli* Gr-4<sup>T</sup>

Features	Chromosome
Length (bp)	2,917,404
Total gene	2696
G+C content (%)	70.5
Genes (coding)	2481
Pseudo genes	162
CDS	2481
rRNA gene	3
tRNA genes	46

**Table 3** Cellular fatty acid profiles of strain Gr-4<sup>T</sup> and phylogenetically related species of the genus *Luteimonas*

Fatty acids	1	2	3	4
<b>Saturated</b>				
C <sub>14:0</sub>	1.2	1.0	0.6	0.9
C <sub>16:0</sub>	4.7	1.8	1.2	3.1
<b>Branched</b>				
iso-C <sub>10:0</sub>	tr	0.8	–	–
iso-C <sub>11:0</sub>	6.6	8.0	18.1	8.1
iso-C <sub>14:0</sub>	4.2	10.6	2.3	3.1
<b>iso-C<sub>15:0</sub></b>	<b>28.6</b>	<b>22.0</b>	<b>24.3</b>	<b>33.6</b>
iso-C <sub>15:1</sub> F	1.1	1.9	2.6	1.2
<b>iso-C<sub>16:0</sub></b>	<b>15.3</b>	<b>13.1</b>	<b>6.2</b>	<b>12.1</b>
iso-C <sub>17:0</sub>	6.0	2.9	5.2	6.7
anteiso-C <sub>15:0</sub>	5.0	3.6	3.2	4.3
cyclo-C <sub>17:0</sub>	0.5	–	1.3	–
<b>Hydroxy fatty acids</b>				
iso-C <sub>11:0</sub> 3-OH	5.6	6.8	14.8	7.6
iso-C <sub>12:0</sub> 3-OH	0.9	0.6	tr	tr
C <sub>16:0</sub> 3-OH	0.9	–	–	–
<b>Summed feature<sup>a</sup></b>				
3; C <sub>16:1</sub> ω7c and/or C <sub>16:1</sub> ω6c	6.7	2.6	2.8	3.2
<b>9; iso-C<sub>17:1</sub> ω9c and/or C<sub>16:0</sub> 10-methyl</b>	<b>11.7</b>	<b>13.7</b>	<b>10.0</b>	<b>7.3</b>

The major fatty acids (> 10%) are in bold type

1, Gr-4<sup>T</sup>; 2, *Luteimonas padinae* KCTC 52403<sup>T</sup>; 3, *Luteimonas terricola* DSM 22344<sup>T</sup>; 4, *Luteimonas arsenica* KCTC 42824<sup>T</sup>. Major components (> 10.0%) are highlighted in bold. The data shown in this table are more than 1% for all strains, tr < 0.5%; –, not determined

<sup>a</sup>Summed features represent groups of two or three fatty acids that could not be separated by gas chromatography (GLC) with the MIDI system

distinguished strain Gr-4<sup>T</sup> from the other recognized species of the genus *Luteimonas* (Table 3).

On the basis of the data and observations described above, it is appropriate to conclude that strain Gr-4<sup>T</sup> should be assigned to the genus *Luteimonas* as the type strain, for which the name *Luteimonas granuli* sp. nov. is proposed.

## Description of *Luteimonas granuli* sp. nov.

*Luteimonas granuli* (gra.nu'li. L. gen. n. granuli of a small grain, pertaining to a granule, from which the type strain was isolated).

Cells are Gram-reaction-negative, aerobic, non-motile and rod-shaped. The colonies grown on R2A agar plate for 2 days are light yellow color, smooth, opaque, circular with regular margins, and 1–2 mm in diameter. Growth occurs between 18–42 °C (optimum, 30 °C), pH 6–8 (optimum, pH 7) and 0–3% NaCl (optimum, without % NaCl). Does not hydrolyze casein, starch, CM-cellulose and DNA. In the API kits (API 20 NE, 32 GN, and API ZYM) system, positive for esculin hydrolysis, nitrate reduction, β-galactosidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, salicin, D-glucose, N-Acetyl-D-glucosamine, and glycogen. Q-8 is the predominant respiratory quinone and iso-C<sub>15:0</sub>, iso-C<sub>16:0</sub> and summed feature 9 (comprising iso-C<sub>17:1</sub> ω9c and/or C<sub>16:0</sub> 10-methyl) are the major cellular fatty acids. The G+C content of the genomic DNA is 70.2 mol%.

The type strain, isolated from the granules used in the wastewater treatment lab scale reactor of in Daejeon, Republic of Korea, is Gr-4<sup>T</sup> (=KACC 16614<sup>T</sup> = JCM 18203<sup>T</sup>).

**Acknowledgements** This research was supported by the project on survey and excavation of Korean indigenous species of the National Institute of Biological Resources (NIBR), by a grant from the Korea Research Institute of Bioscience & Biotechnology (KRIBB) Research Initiative Program and by Korea Research Fellowship of Korea (NRF) funded by the Ministry of Science and ICT (Project No. 2019H1D3A1A02070958). (KRF) Program through the National Research Foundation.

**Author Contributions** Conceived and designed the experiments: MZS, WTI. Performed the experiments: MZS, WTI. Analyzed the data: SYK, JHY, WTI. Wrote the paper: MZS.

## Compliance with Ethical Standards

**Conflict of interest** The authors declare that there is no conflict of interest.

**Ethical Approval** This article does not contain any studies with human participants or animals performed by any of the authors. Moreover, all authors read and approved the final manuscript.

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